Sex-Differences in the Prophylactic Effects of Ketamine as an Antidepressant

Undergraduate Research Thesis

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Abstract:

Stress-related mood disorders are more frequently observed in women. Females mice have been shown to have a more sensitive prefrontal GABAergic system (e.g. increased parvalbumin – PV – expression) following exposure to Unpredictable Chronic Mild Stress (UCMS), than males. The NMDA receptor (NMDA-r) antagonist ketamine is known to induce sex-dependent antidepressant like effects following exposure to stress in mice, possibly through NMDA-r expressed onto PV-expressing neurons. These findings suggest that sex-specific vulnerability to stress might be associated with differential sensitivity of NMDA-r on PV cells. To test this hypothesis, ketamine was given prophylactically in mice exposed to UCMS. Male and female C57bl/6 mice were assigned to a Control group, or a UCMS group. Subjects within each treatment group received a dose of ketamine (10mg/kg) or vehicle (0.9% saline) intraperitoneally (n=8/group) a week before the experimental period. Mice were then tested in the Open Field (OF) and Forced Swim tests (FST) to measure anxiety- and depressive-like behavior, and in the Object Recognition Test (ORT) to analyze cognitive functions, and their brains were collected after the behavioral testing period. To evaluate ketamine's short term effects, a second group of 10 males and 10 females received an equal dose of ketamine, and their brains were collected and analyzed 7 days later. Brains were assessed for mRNA expression of NMDA-r glutamate receptor subunit proteins Grin 1 and Grin 2a, and PV. RT-PCR analyses reveal sex-specific effects of ketamine on mRNA expression of Grin1, Grin 2a, and PV in the PFC that suggest a better drug response in males, than in females. Similarly, behavioral data show that ketamine prevents the depressive-like phenotype in UCMS-exposed males, but not females. Ketamine did not prevent UCMS-induced anxiety-like phenotype in males and females, and did not prevent induced cognitive deficits in males. Altogether, our data demonstrate that inactivation of NMDA

receptors prior to stress might provide resilience to stress-induced depression in males, but not in females, suggesting sex-specific molecular mechanisms to stress vulnerability.

Introduction:

The word ‘stress’ is one that has become common place in day to day living. Students in high school to college commonly express their concern with academic performance by using the word to describe their current state of being. Even following graduation, stress is still seen as an aspect of the professional life with adults stressing about meeting deadlines, reaching quotas, or attaining their next career goal. The historical study of stress led to the introduction and definition of two distinct forms of stress. In 1936, endocrinologist Hans Selye published a short article in *Nature* with the purpose of increasing knowledge about stress. Of the many important details discussed in that article, his distinction between negative stress, distress, and positive stress, eustress, was a key stepping stone in increasing Psychology and Neuroscience understanding of stress (Szabo S, 1998).

In present day, eustress is still used to describe a cognitive response to a stressful stimulus that is positive, healthy, and often serves as a motivation for productive behavior. Conversely, distress is a negative response to stressors which often leads to maladaptive behavior and physiological cascades in the body. Eustress and distress are commonly associated with chronic stress, which describes a state of prolonged exposure to external or internal stressful stimuli. Current Neuroscience and Psychology research reveals that exposure to chronic stress is often positively correlated with an increased risk for mood disorders, including anxiety and major depressive disorder of (MDD) (Gold and Chrousos, 2002; Dunman and Monteggia, 2006). Clinical diagnosis of MDD is usually conducted in accordance with regulations outlined by the Diagnostic and Statistical Manual of Mental Disorders (DSM) (Figure 1).

Additionally, individuals are known to have varying vulnerabilities to stress, with females being more likely to develop these emotional disorders following stress than males (Kornstein, 1997; Kendler, 1998). In regards to comorbidity, depression and anxiety are also frequently observed together in women than in men (de Graff et al., 2002; Schoevers et. al., 2003). However, the exact mechanism underlying the increased vulnerability to stress-related emotionality disorders in women remains unknown.

Current neurological understanding of emotionality disorders reveals a hypothesis that symptoms associated with such disorders often stem from malfunction in the prefrontal cortex (PFC). The PFC is the part of the cerebral cortex located at the forefront of the brain. Over the course of research related to its study, it has been deduced that the PFC plays a significant role in the development of a person's personality. Additionally, another important term frequently used to describe the responsibilities of the PFC is its role in executive functioning. Executive functioning refers to a collection of cognitive processes- i.e. those involved in planning, control of attention, working memory, reasoning, problem solving, control of inhibition- that are necessary to one's parenting of his or her own behavior, as well as executing behaviors that are necessary for the attainment of individual goals.

As such, Neuroscience and Psychology research focused on understanding the complexity of the PFC has often led to the elucidation of previously misunderstood characteristics of emotionality. For example, a study published by Baxter et al. in 1989, reveals hypoactivation in the dorsolateral PFC as a distinct characteristic of depression. Direct target of this physiological characteristic by brain stimulation was proven to improve a percentage of symptoms commonly observed in clinically diagnosed depressed and anxious humans (Mayberg et al., 2005; O'Reardon et al., 2007).

This research on the PFC has not only revealed its role in emotionality disorders, but has also drawn attention to its sensitivity to stress. A paper published by Covington et al. in 2005 examines chronic social defeat stress, cocaine intake, and the role of the amygdala and PFC. Data from this study reveal that following a 60-day period of social defeat, levels of zif268 mRNA gene expression were increased in the central and medial amygdala of subjects (Long-Evans rats). Conversely, the levels of zif268 mRNA gene expression were decreased in the PFC. In this study, zif268 levels were measure as an indicator of cellular activity. The cellular activity in nuclei of the amygdala and PFC is commonly studied through the measurement of immediate early genes (IEG) expression (Herdegen and Leah, 1998). An IEG and a member of the krox family, Zif268 expression is typically correlated with the functional level activity in the brain (Worley et al, 1991). The activity of Zif268 is not only affected by changes in membrane potentials, but also by administration of drugs and exposure to stressful stimuli and experiences (Worley et al, 1991, Honkaniemi et al, 2000; Mutschler et al, 2000). As such, this paper as well as a subsequent study published by the same lab in 2010 were key in drawing Neuroscience research attention to stress induced abnormal activity in the PFC which also correlated with depressive- and anxiety-like behaviors.

Regarding the difference in vulnerability observed in females and males, and the relation of this phenomenon to the PFC, sex-influenced effects of stress on the PFC have also been consistently found. One such evidence is revealed in analysis of prefrontal neurons projecting the amygdala. In stressed females, there is a different pyramidal cell morphology when compared to the female control although in general estrogen was revealed to promote difference in cell morphology even in unstressed females (Shansky et al., 2010). These neuronal structural changes include differences in dendritic length, and spine density. Conversely, male subjects did not

display such structural changes (Shansky et al., 2010). In fact, medial prefrontal cortex (mPFC) neurons in male rats projecting to the basolateral nucleus of the amygdala were found to be resilient to the dendritic remodeling induced by stress.

Additional studies conducted in rats provide evidence that further support the notion of a more chronic stress sensitive PFC in female rats, than in male rats (Garrett and Wellman, 2009). Although chronic stress induces morphological changes in the PFC neurons of both males and females, the nature of this change was found to vary based on the subject's sex. In male rats, exposure to chronic stress (3 hours of restraint daily for a week), decreased apical dendritic branch number and length. However, in females, stress increased apical dendritic length (Garrett and Wellman, 2009).

Further research to confirm if this observed sex-specific stress induced responses were influenced by estrogen was conducted in a second experiment which included female rats that received either ovariectomy with or without 17-beta-estradiol replacement or sham ovariectomy. Analysis of the results of this research reveal that both the ovariectomized and sham-operated rats with the implants of estradiol displayed stress-induced increases in apical dendritic material (Garrett and Wellman, 2009). However, the ovariectomy without estradiol replacement prevented the stress-induced increase (Garrett and Wellman, 2009). Together, the results of both experiments conducted by Garrett and Wellman suggest that the stress-induced increase in apical dendritic material in females is estradiol-dependent.

It is therefore evident that interaction between the PFC and the amygdala might be responsible for the observance of sex differences in stress- related emotionality disorders. As part of its functions, the PFC is known to enact effects on the limbic regions in a top-down fashion. These limbic regions include the amygdala and the striatum (Sotres-Bayon et al., 2004; Kumar et

al., 2013; Adhikari et al., 2015). Through this top down regulation of the limbic regions, the PFC thus impacts perception and processing of motivation, anxiety, and rewards (Lalumiere, 2014). As such, current Neuroscience and Psychology research suggests that this top-down processing is the medium by which the PFC regulates emotional, and even inhibits behavior that might be deemed as socially unacceptable.

It is reasonably hypothesized that stress-induced changes to the PFC affect this downstream regulation. Since stress induces sex-dependent changes in the PFC, it is also reasonable to hypothesize that these sex-dependent differences in response to chronic stress are the reason for sex-dependent differences in stress- induced emotionality disorders. However, since stress's effects on the prefrontal cortex are primarily evident in the resulting hypoactivity observed in the PFC following stress, elucidating the mechanism by which this hypoactivity is induced was a critical step in catapulting Neuroscience and Psychology stress research into its next phase.

The makeup of cells in the PFC is shown to be diverse as it consists of both excitatory pyramidal cells and inhibitory GABAergic cells (Steketee, 2005). Traditionally, it had been suggested that a down regulation of the GABAergic system was implicated in observed phenotypes of depression and anxiety. This traditional believe led to the development of many GABA-agonists or combination therapies with benzodiazepines as the treatment for depression and anxiety (Johnson, 1985; Furukawa et. al., 2002). However, over the past few years, there has been a gradual transition in perspective as recent studies reveal an upregulation in the GABAergic system (Michels et al., 2014; McKlveen et al., 2016), and not necessarily down regulation of excitatory pyramidal cells, is the reason for the observed stress induced anxiety and depressive behavior. For example, a recently published paper by Coutellier Lab indicates that

exposure to chronic stress induces sex-dependent changes in the prefrontal GABAergic system that is more prominent in females than males (Shepard et. al., 2016). In accordance with these changes in the GABAergic signaling, sex-specific changes were also observed in the analysis of the top-down processing pathway between the PFC and limbic regions, and their associated emotional and behavioral manifestations. More specifically, the level of parvalbumin (PV) expression (a calcium-binding protein expressed in a specific sub-type of GABAergic interneurons) in the PFC of females following UCMS was higher than in males, and significantly correlated with their level of anxiety.

The goal of this experiment was to further our understanding of these sex-dependent stress-induced prefrontal GABAergic disturbances, and how they relate to PFC-amygdala alterations, and increased emotionality in females. Ketamine, an NMDA-receptor antagonist, is believed to exert its anti-depressant action through NMDA-receptor expressed onto PV cells (Zhou et al., 2015). Furthermore, studies conducted with human patients, and in animal models of depression using ketamine have been shown to display both rapid and prolonged anti-depressant effects of ketamine (Skolnick et al., 2009). Historically, Neuroscience research has revealed that a subject's response to an antidepressant drug is highly sex-dependent (Sloan and Kornstein, 2003; Marcus *et al*., 2005; Pitychoutis *et al*., 2010; Dalla *et al*., 2011; Pitychoutis *et al*., 2011 ; Pitychoutis *et al*., 2012). Specifically, studies focused on ketamine administered as a treatment for depressive-like symptoms in mice have shown that response to treatment is sex-dependent (Carrier and Kabbaj, 2013). Such findings suggest that sex-dependent differences in vulnerability to stress might be due to stress-induced increased sensitivity of NMDA-receptors onto PV cells in females.

Two papers have been published since 2016 examining the potential of prophylactic ketamine. A paper published by Brachman et al., 2016, utilized a chronic social defeat (SD) stress model, learned helplessness (LH), and a chronic corticosterone (CORT) model in male mice, to assess if ketamine could protect against depressive-like behavior. Mice in the Brachman's study were administered a single dose of saline or ketamine (30 mg/ kg) and then 1 week later were subjected to 2 weeks of SD, LH training, or 3 weeks of CORT. The second study, conducted by McGowan et al., 2017 sought to determine the best time to administer ketamine relative to fear experience. Using a dose of 30 mg/kg of ketamine, the McGowan study determined that ketamine's ability to buffer a fear response is optimal when administered prophylactically, and a week before exposure to stressor. Our experiment varies from the Brachman and McGowan studies as it explores the prophylactic potential of ketamine in both male and females. Additionally, our experiment does not just examine the behavioral manifestations of ketamine's effects, but also considers its effects on a molecular level by specifically examining as they pertain to NMDA-receptors and PV cells in the PFC. Using a pharmacological approach, our experiment was aimed at showing that increased prefrontal inhibition following chronic stress in females is directly responsible for heightened anxiety- and depressive-like behaviors.

Diagnostic Criteria

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.
   - **Note:** Do not include symptoms that are clearly attributable to another medical condition.

   1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty, hopeless) or observation made by others (e.g., appears tearful). (**Note:** In children and adolescents, can be irritable mood.)
   2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).
   3. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. (**Note:** In children, consider failure to make expected weight gain.)
   4. Insomnia or hypersomnia nearly every day.
   5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
   6. Fatigue or loss of energy nearly every day.
   7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
   8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
   9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

Figure 1: The above figure illustrates the possible symptoms required for a diagnosis of Major Depressive Disorder as detailed in the Diagnostic and Statistical Manual for Mental Disorders, Fifth Edition (DSM V) (American Psychiatric Association). MDD is an emotional disorder commonly induced by stress.

Research Overview:

Hypothesis:

I hypothesize that sex- specific vulnerability to stress might be associated with differential expression of NMDA-r on PV cells.

Purpose:

To investigate this hypothesis, in the present study, ketamine was given prophylactically to mice exposed to chronic stress. Sixteen male and female C57bl/6 mice were randomly assigned to a Control group, or a UCMS group. One week prior to the beginning of the experimental period, subjects within each treatment group were given a dose of ketamine (10mg/kg) or vehicle (0.9% saline) intraperitoneally (n=8/group). The relevance of this study is therefore to identify a molecular pathway and mechanism that can explain sex-dependent differences in stress vulnerability. Identifying this pathway and its relationship to ketamine and other NMDA-r antagonists can lead to new pharmacological and behavioral treatments for depression and anxiety populations, especially in those most susceptible to stress.

Methods:

Experimental Subjects:

Mice of the strain C57Bl/6j (B6) were used. These mice were adults (8 weeks) and were ordered from Jackson Laboratory (Maine, US). A total of 84 mice (64 for the analysis of ketamine's Long Term Effects (LTE) and 20 for the analysis of ketamine's short term effects (STE) were used in this experiment. Upon delivery to Psychology Building facility located on Ohio State's Main Campus, mice were allowed a 6 day period to habituate to the facility's colony room located in the building's basement, and were maintained on a 12 h reverse light-dark cycle with access to food and water *ad libitum*. Following this habituation period, the 64 mice belonging to the LTE phase of the experiment were randomly divided into experimental groups, and treatment groups. In this experiment, there were three variables: sex, drug treatment, and experimental treatment. For each of these variables, there were to components. The two components of the sex variable were male and female. The two components of the drug treatment were ketamine (10 mg/kg) or vehicle (0.9% saline); which were administered intraperitoneally. Finally, the two components of experimental treatment were control (handling of the mice) or UCMS. With three variables, and two components of each variable, this experiment thus consisted of a 2x2x2 model and consequently, 8 total groups. The 8 groups were as follows: Vehicle + Control + Male, Vehicle + Control + Female, Ketamine + Control + Male, Ketamine + Control + Female, Vehicle + UCMS + Male, Vehicle + UCMS + Female, Ketamine + UCMS + Male, Ketamine + UCMS + Female.

Experimental Schedule Overview: LTE

Subjects belonging to the analysis of ketamine's LTE were habituated to the colony room for 6 days. On the seventh day (Fig. 2), subjects were either injected intraperitoneally with ketamine (10 mg/kg in 0.9% saline) or with vehicle (saline). This day was considered as Day 1 (D1). From D2-D6 (Fig. 2), subjects could rest. D7 was the first day of experimental treatments and subjects were either placed in UCMS, or handled for a duration of 1 to 2 minutes with care. The experimental treatment phase lasted for 29 days (Fig. 2 D7- D35).

Following the experimental treatment phase, subjects began behavioral testing on D36 (Fig.2 D36). The first day of behavioral testing was comprised of the Open Field Test (Fig. 2. D 36). On the second day, mice were tested using the Object Recognition Test (ORT) (Fig. 2. D 37-38). Day four was the final day of behavioral testing and mice were placed in the Forced Swim Test (FST) (Fig. 2. D. 39). Following behavioral testing, mice brains were collected on Day 45.

Experimental Schedule Overview: STE

To observe the short-term effects of ketamine, 20 mice (10 males and 10 female subjects of the C57Bl/6j (B6) were included in this study. These animals were also obtained from Jackson Laboratory (Maine, US). Subjects belonging to the analysis of ketamine's STE were habituated to the colony room for six days. on the seventh day (Fig. 3 D1), subjects were either injected intraperitoneally with ketamine (10 mg/kg in 0.9% saline) or with vehicle (saline). Mice were then kept in the colony room for 7 days with access to food and water and maintained on a 12 h reverse light- dark cycle. Brain collection for these subjects occurred on Day 8. Figure 3 details a schedule for the STE subjects.

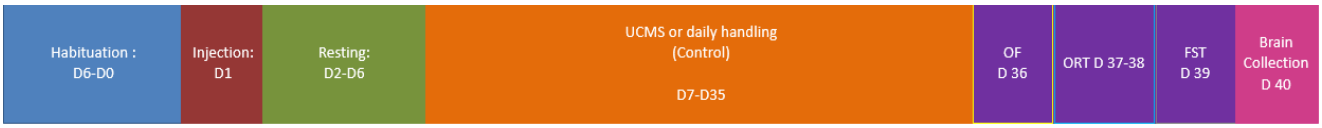

Figure 2: *Sex-Differences in the Prophylactic Effects of Ketamine as an Antidepressant* experimental timeline for LTE group

Figure 3: *Sex-Differences in the Prophylactic Effects of Ketamine as an Antidepressant* experimental timeline for STE group

Unpredictable Chronic Stress Model

Mice in the experimental UCMS group were exposed to a random stressor a day for 28 days. According to the UCMS paradigm, the stressors the mice were exposed to were chosen in such a way to be unpredictable and random. As such, administration of stressful stimuli was not only randomized in terms of the day of the week that subjects were exposed to a specific stressor, but also in terms of time of the day. Example of stressors used include absence of nesting material in the subjects' cage for an 8-hour period, tilting the cage at 90-degree angle for an 8-hour period, restraining mice in light conditions for four minutes, or restraining subjects in dark conditions for 8 minutes. A chart of possible stressors is shown on the following page in (Table 1). Additionally, a sample calendar (Table 2) has been provided to depict how these stressors were scheduled. All mice stressors and behavioral analyses were conducted between 8 am and 8 pm, in order not to interfere with the light cycle regulated in the colony room.

STRESSOR	DURATION	DESCRIPTION	LITERATURE
CAGE TILT	6 hours	Subject's cages were mounted at a 45 ° angle	Willner et al. 1992 Ibarguen-Vargas et al. 2008 Surget et al. 2008
NO BEDDING AND NESTING MATERIAL	8 hours	Mice were placed in an empty cage devoid of any nesting or enrichment material	Ibarguen-Vargas et al. 2008 Surget et al. 2008
BRIGHT RESTRAINT	4 minutes	Restraint stress for 4 minutes in a lit room (bright light)	Nollet et al. 2013
DARK RESTRAINT	8 minutes	Restraint stress for 8 minutes in a dark room.	Nollet et al. 2013
NO NESTING MATERIAL	24 hours	All nesting cubes were taken from subject's cage.	Willner et al. 1992 Ibarguen-Vargas et al. 2008 Surget et al. 2008

Table 1: The chart above displays stressors used in this experiment. In the further most right column, similar experiments using the same or similar UCMS stressors are cited.

	SUN	MON	TUES	WEDS	THURS	FRI	SAT
STRESS		Dark Restraint (8 minutes)	Cage Tilt (6 hrs)	Empty Cage (8 hours)	Bright Restraint (4 mins)	Remove nesting material	
STRESS	Cage Tilt (6 hours)	Bright Restraint (4 minutes)	Remove nesting material (24 hours)	Empty cage (6 hours)	Dark Restraint (8 minutes)	Cage Tilt (6 hrs)	Bright Restraint (4 mins)
STRESS	Empty Cage (8 hours)	Dark Restraint (8 minutes)	Cage Tilt (6 hrs)	Remove nesting material (24 hours)	Bright Restraint (4 mins)	Empty Cage (8 hours)	Remove nesting material
STRESS	Change Cages for group in preparation for behavioral testing.						

Table 2: Displayed above is a sample calendar for mice in the UCMS treatment condition.

Behavioral Tests:

Open Field Test:

The open field test was administered as a measure of subjects' anxiety like behavior and activity. The open field testing arena is a 40 by 40 cm square arena with Pyrex glass walls and a grey base. Prior to the beginning of testing, the grey base is divided into 16 virtual squares which are marked using white tape. Hoisted steadily above the arena is a camera which allows for the offline analysis of the animal's behavior. As a behavioral assay, the ratio of time spent in the center of the arena versus by the walls, and the total number of squares crossed are used as indicators of anxiety-like behavior and locomotor activity, respectively.

Open field testing was conducted during the dark phase of the light cycle of the mice. Prior to testing, subjects were given an hour to habituate to the testing room. After habituation, each mouse was permitted to explore the arena for 10 minutes. Testing was conducted in dim overhead light conditions. At the conclusion of these 10 minutes, the subject was placed back in its cage. The arena was then cleaned with 70% ethanol. Scoring for this test was completed using Noldus Information Technology software. Data generated from the software's analysis were then compiled and analyzed using SPSS and Graph Pad Prism Software.

Forced Swim Tests:

A widely acknowledge assay for analysis of depressive-like behavior, the forced swim test is often used as a model of helplessness, which is observed in relation to a subject's time spent immobile.

The test is conducted in a glass cylinder (height: 30 cm; diameter: 15cm). On the day of testing, the apparatus is filled with water to a temperature of 24-25^{o}C. Each subject was placed in the water filled apparatus for 6 minutes. Following testing, the mice were promptly removed from the cylinder and placed back in their home cage which was placed on a heating pad to warm the subject and get him/ her dry.

All trials for the forced swim test were recorded using a video camera that was mounted to allow a good angle of the side of the transparent cylinder to adequately perceive mice

behavior. Analysis was completed offline and the latency to first immobility and the total time spent immobile during the 6-minute duration were used as markers for depressive-like behavior.

Immobility was defined as complete absence of movement, aside for movement involved in respiration.

Novel Object Recognition Test:

Analysis of cognitive abilities were performed through the Object Recognition Test (ORT). Object recognition testing is conducted over a period of 3 days: Day 1: Habituation to Arena without objects (10 minutes), Day 2: Part 1 Habituation without objects (5 minutes), Part 2: Training with objects (10 minutes), Day 3: Novel Object Recognition (10 minutes).

Subjects were habituated to the testing room for at least an hour during each testing day. ORT was conducted during the dark phase of the subjects' light cycle. At the conclusion of this habituation period, the testing phase began. Since the arena used for this ORT is the same as the arena used for open field testing, Open Field Test was used as Day 1 of ORT to minimize the duration and stress of testing period. Following the conclusion of Day 1, subjects were transported to the colony room.

Testing resumed the following day and was broken down into two parts. Similar to Day 1, Part 1 of Day 2 involved the subject's habituation to the arena during the first 5 minutes. Following these 5 minutes, two identical objects were placed in the arena to allow for each subject to associate these novel objects to the arena. The two identical objects used were either two glass bottles or two tubes weighed down by pennies. Objects were assigned to each mouse randomly as to improve the validity of the test. The subjects were allowed 10 minutes to explore the two identical objects (= training phase). At the end of 10 minutes, the subject was moved from the arena, and the arena along with the two identical objects were cleaned with 70% ethanol in between subjects.

Exactly 24 hours after the training phase (Day 2), subjects completed Day 3: testing day. Following a one hour habituation period to the room, subjects were placed in the arena which contained non-identical objects (one glass bottle and one tube of pennies). Each subject was

given 10 minutes. Following the completion of each subject's testing, the arena and objects used were cleaned with 70% ethanol.

All days of testing were scored by an experimenter on a desktop computer using three timers apart from Day 1 which was scored as the Open Field test described above. Scoring of testing days evaluated the time subject spent exploring or sniffing objects in the arena. Time spent exploring was defined as the mouse's nose being directed toward the object (<1cm away from the object), or touching the object with front paws. Climbing or seating on the object was not scored as exploration.

Tissue Collection:

Twenty-four hours after behavioral testing, subjects were euthanized and their brains were collected. 16 male and 16 female mice from LTE; and 5 males and 5 females from the STE, were anesthetized with isoflurane and their brains collected to assess the mRNA expression of (Grin 1, Grin 2a and parvalbumin – PV). Brains that were not perfused were collected and flash-frozen on dry ice. All brains were stored at 80^{o} C until dissection. These brains were dissected in a cryostat regulating cold temperature (-25^{o}C) on dry ice: the PFC was collected according to the Mouse Brain Atlas of Franklin and Paxinos (2008).

RT- qPCR Protein Analysis:

Homogenization:

Dissected PFC samples were weighed in order determine buffer to sample ratio for homogenization step. Samples were then homogenized for 10 seconds using QiaZOL buffer under a hood in culture tubes according to the ratio 10,000ul Qiazol/gram of tissues. All samples were kept on dry ice and homogenization was performed at room temperature and as quickly as

possible and under the most sanitary conditions to avoid cross sample contamination. All tubes and material used were treated to be RNA- nuclease free. Generated lysate samples were then stored at -80 ^{O}C in between progression to the next step.

RNA Extraction:

The following steps were completed for each sample. Lysate samples generated from the homogenization step were placed on the bench top for 5 minutes in order to allow for the dissociation of nucleoprotein complexes. Chloroform was then added, and the solution was shaken vigorously for 15 seconds. This solution was then placed on the bench top for 2-3 minutes. Following this period, the solution was centrifuged at 4^{o}C. Centrifugation resulted in the separation of the solution into 3 phases: an upper, colorless, aqueous phase containing RNA, a white middle layer phase, and a lower, more red organic phase. The upper aqueous layer was transferred to and RNase- Free tube to which a volume of 70% ethanol was added. The sample was then vortexed. Steps were taken to obtain 2 portions of a flow through which was discarded. A buffer was added to the remaining sample which was then centrifuged in order to wash the membrane. The flow through obtained from this step was also discarded.

DNase Digestion

DNAse Digestion was completed RNase-Free DNase Set. The stock solution was prepared and then 10 µl of DNase I stock solution was added to 70 µl Buffer RDD. The sample was mixed via inversion, and then centrifuged in order to obtain residual liquid from the sides of the tube. DNase I incubation mix (80 µl) was then added directly to the RNeasy spin column membrane, and placed on the benchtop (20–30°C) for 15 min.

Final Sample Preparation

A buffer (Buffer RW1) was added to the sample and centrifuged for 15 s at 8000 x g (10,000 rpm), and the flow through was discarded. 500 µl of Buffer RPE was added to the sample to, and centrifuged for 15 s at 8000 x g (10,000 rpm) to wash the membrane, and the flow-through was discarded. 500 µl of Buffer RPE was added to the sample and washed again for 2 min at 8000 x g (10,000 rpm). The purpose of this rigorous washing step was to ensure that no ethanol contaminates the RNA elution. All flow through was discarded. The sample was then centrifuged again to eliminate traces of Buffer RPE. Following this centrifugation, 40ul of RNase-free water was added to the sample. Elution of the RNA was completed by centrifuging the sample for 1 min at 8000 x g (10,000 rpm). The obtained 40ul of water was to be used as the mRNA sample which was placed immediately on wet ice.

Quantification of mRNA purity

Quantification of the m RNA sample purity was completed using BioTek technology devices. A generated excel file Excel file was used to determine the dilution of samples needed to generated cDNA samples.

Real Time qPCR analysis:

Analysis of cDNA samples was completed for mRNA expression of the following targets: Grin 1 (NR1), Grin 2a (NR2), and parvalbumin- PV. These target cDNA and the reference target (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Primer sequences were as follows: *GAPDH*: (Fwd.: 5'- CAT GGC CTT CCGTGT TCC T -3'; Rev.: 5'- TGA TGT CAT CAT ACTTGG CAG GTT -3'; Efficiency: 92.10 %, Temperature: 57.5^{o} C) *Grin 1*: (Fwd.: 5'-CAG GAG CGG GTA AAC AAC AGC AAC -3'; Rev.: 5' GAC AGC CCC ACC AGC AGC

CAC AGT -3'; Efficiency: 100.20%; Temperature: 57. 5^{o}C), *Grin 2a* (Fwd.: 5'-AGC CCC CTT CGT CAT CGT AGA -3'; Rev.:5'-CAG AAG GGG AAA CAG TGC CAT TA-3'; Efficiency 103.50, Temperature: 60), PV (Fwd. 5'-AGC GTC TTT GTT TCT TTA GCA G-3'; Rev. 5'-ATG AGGTGA AGA AGG TGT TCC-3'; Efficiency 92.60%, Temperature: 57.5). Each sample was run in a triplicate for each primer. The conditions used for PCR were: 95 ^{o}C for 30s and 40 cycles of PCR (denaturation: 95^{o}C for 5s, annealing and/or extension: 60^{o}C for 30s). Analysis of data was completed using the comparative Ct (cycle threshold) method.

Statistical Analysis:

Analysis of male and female data was completed for significance using Two Way Analysis of Variance (ANOVA) via the statistical program Prism 7 (Graph Pad Software Inc., La Jolla, CA, USA) or a T- test when applicable. Post-hoc tests (when necessary) were completed using Tukey's Test and Bonferroni Test on the same program. The p-value used to determine significance was (p-values < 0.05). Analyzed data are depicted as mean ±standard error of the mean (SEM).

Results:

Behavioral Analyses:

Open- Field Test

Our data reveal differences between the effects of UCMS on male and female performance in the open field test. Analysis of total locomotion time reveals a main effect of UCMS in males (Fig. 4A; $p=0.0015$). Analysis of total locomotion data for female subjects show a main effect of stress on female locomotion (Fig. 4B; $p<0.0001$). Additionally, a trending effect of ketamine's ability to rescue the normal phenotype was observed in females (Fig. 4B; $p=0.0600$).

Analysis of big center data for male mice reveal no main effect of experimental or drug treatments. Analysis of big center data for female subjects displays a main effect of UCMS on time in big center (Fig.4D; $p= 0.014$). Such behavior evidences stress induced anxiety-like behaviors in female subjects. There was no effect of the ketamine treatment in females.

Open Field Test:

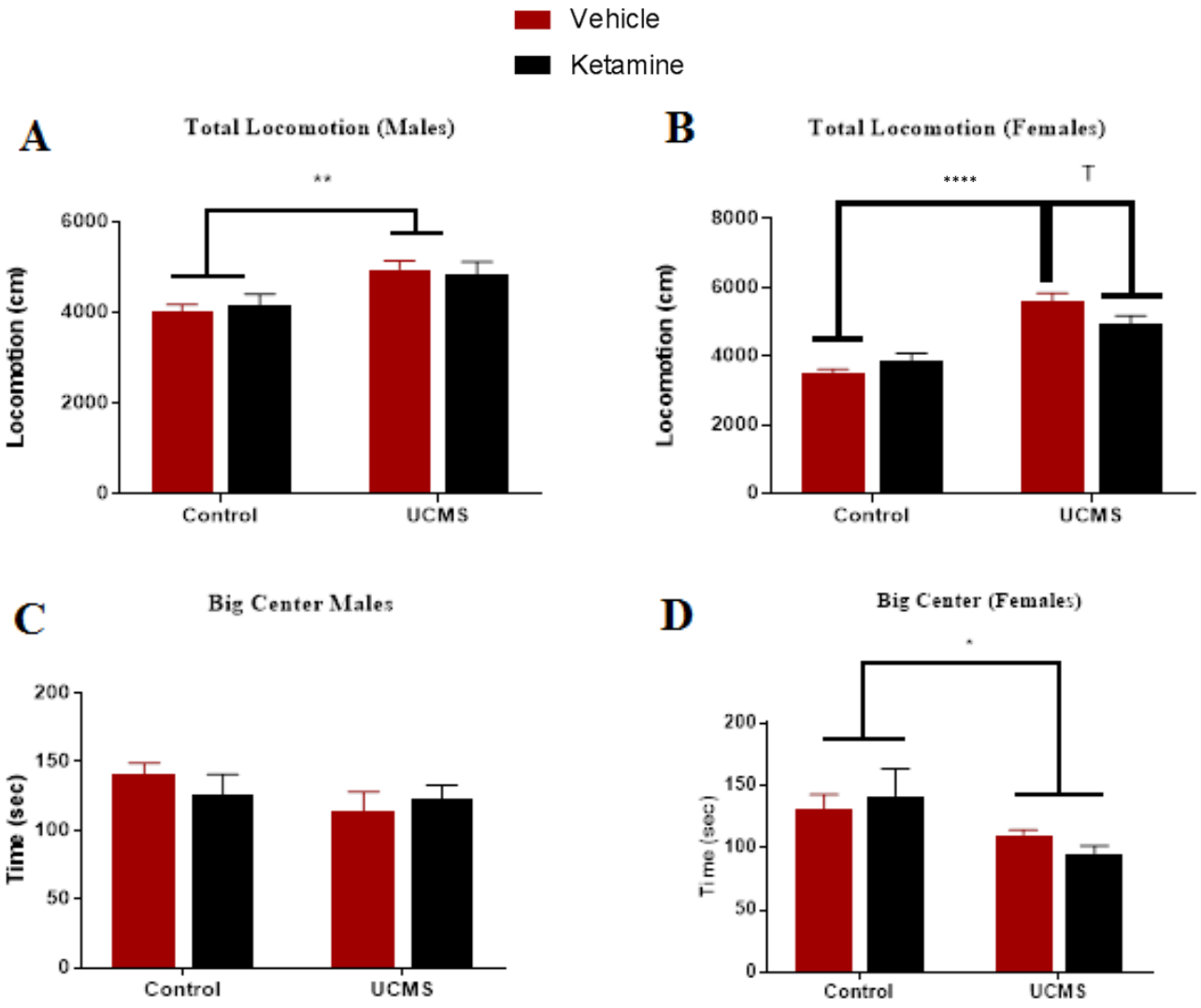

Figure 4: Both males and females display significant hyper-locomotion following stress (Fig. 4A p= 0.0015, Fig. 4B p<0.0001), with UCMS females displaying a trending effect of the rescue of the phenotype with ketamine (p=0.06). Only females displayed anxiety-like behaviors following stress which was not rescued by drug treatment (Fig. 4D)

Object-Recognition Test

The results of the Object Recognition Test are represented in Figure 5. In male mice, there was a main effect of drug treatment. They show that ketamine significantly reduces the ability of male mouse subjects to discriminate between the familiar and unfamiliar objects during testing day (day 3) of the ORT (Fig. 5A; $p= 0.035$)

In females, neither exposure to UCMS nor treatment with ketamine had an impact on ability to discriminate between familiar and unfamiliar object during day 3 of ORT.

Object Recognition Test:

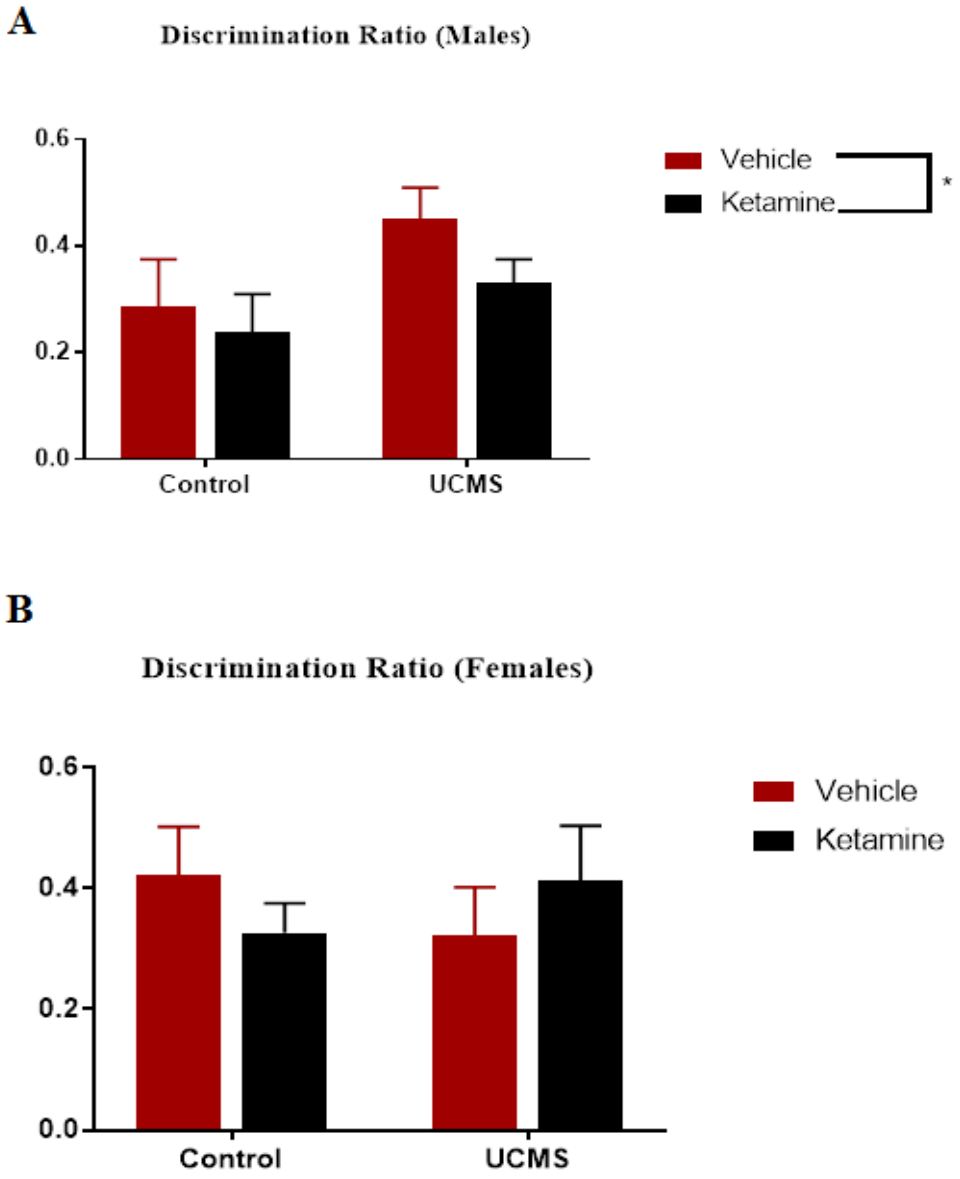

Figure 5: Data show ketamine significantly decreases male subjects' ability to discriminate between familiar and unfamiliar object during day 3 of ORT (Fig. 5A p=0.035). No significant or similar effect detected in females (Fig. 5B).

Forced Swim Test

Analysis of data gathered from FST display significant or trending differences in depressive like behavior for both and females following exposure to UCMS. In males, a main effect of drug treatment in subjects who were exposed to stress was observed. Ketamine treated subjects display a significant increase in latency to first immobility indicating a rescue of the depressive phenotype (Fig. 6A; $p= 0.007$). Analysis of total time immobile in males reveals a main effect of stress which was rescued by ketamine treatment in UCMS subjects (Fig. 6C; $p=0.04$)

Similar to males, females exposed to UCMS treatment displayed higher depressive-like behavior. Analysis of their latency to first immobility reveal a trending effect of UCMS subjects having overall higher latency to first immobility than controls (Fig. 6B; $p=0.07$). Analysis of female subjects' total time spent immobile displays UCMS subjects with significantly increased total time immobile (Fig.6D; $p=0.025$). However, unlike in males, treatment with NMDA-r antagonist, ketamine, did not normalize the UCMS induced phenotype.

Forced Swim Test:

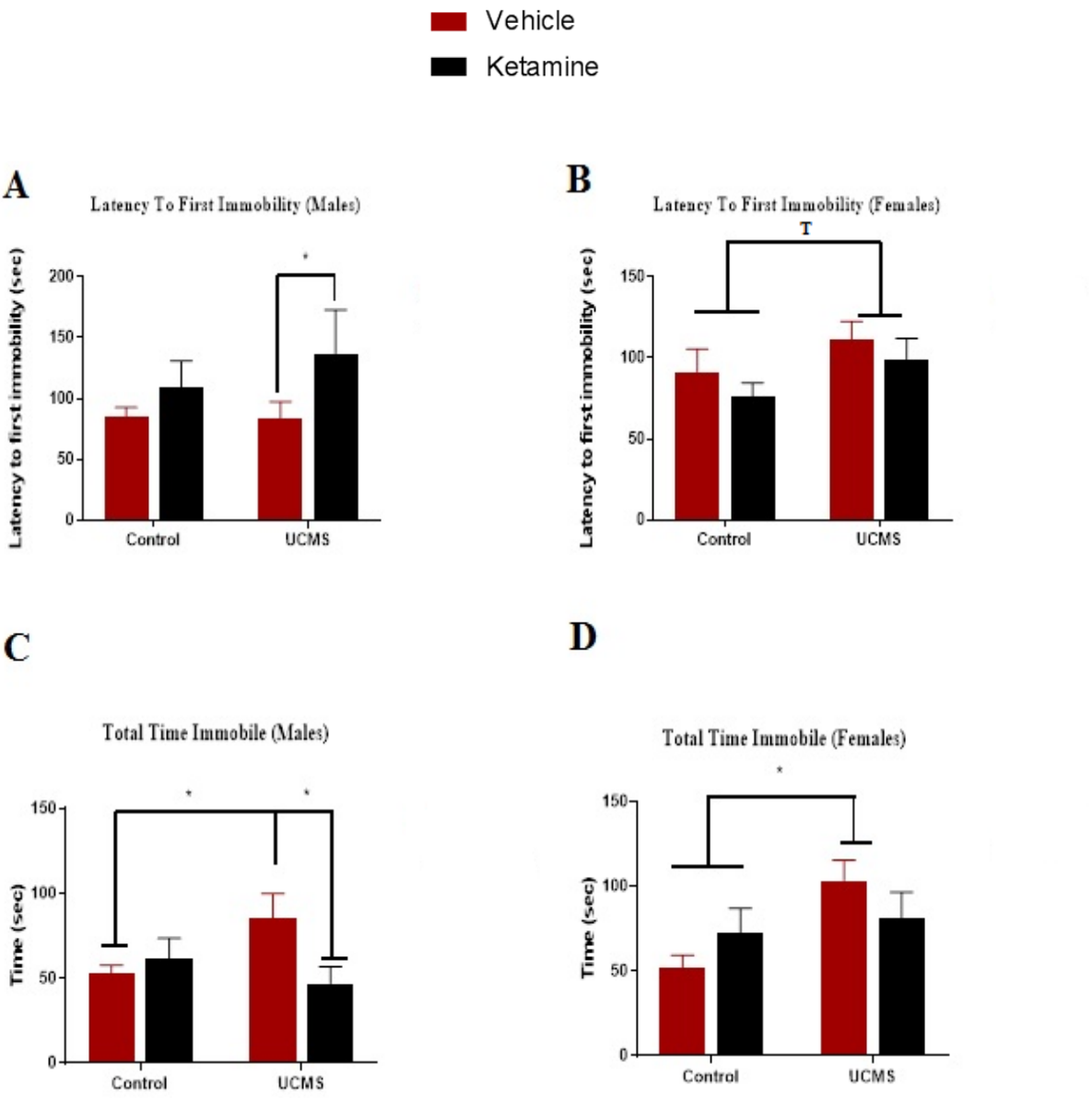

Figure 6: FST data suggests a rescue of the depressive phenotype in males injected with ketamine as shown by normalization of latency to 1st immobility (p=0.007 – Fig. 6A) and time spent immobile (p=0.04 – Fig. 6C). Female data show a similar influence of treatment on phenotype, with UCMS subjects exhibiting more depressive-like behavior than the control group. However, unlike in males, the unstressed phenotype was not rescued by ketamine (Fig. 6B p=0.07, Fig. 6D p=0.025).

RT- qPCR Analyses:

Effects of ketamine on mRNA expression after exposure to UCMS:

Analysis of Grin 1 mRNA expression reveals a significant main effect of stress on protein expression in males that was not rescued by ketamine (Figure 7A: p= 0.0009). Grin 2a mRNA expression reveals a similar significant main effect of stress in males (Figure 7C: p <0.0001). In addition, analysis of mRNA expression for Grin 2a also shows a main effect of ketamine, with lower levels of protein expression in subjects treated with the drug (Figure 7C: p=0.0031). PV mRNA expression was significantly decreased in UCMS males (Figure 7E: p= 0.0326), indicating a main effect of stress. In addition to stress' impact on PV expression, a significant decrease in PV mRNA expression was observed in ketamine treated subjects (Figure 7E: p= (p=0.00114)).

Female data reveal no significant main effects of stress or drug treatment on Grin 1 mRNA expression (Figure 7B). Analysis of Grin 2a mRNA expression in females reveals no significant effects of stress or drug treatment on protein expression (Figure 7D). PV mRNA expression data also show no significant impact of stress on protein expression, and show no significant effects of drug treatment on PV mRNA expression (Figure 7F).

Effects of ketamine on mRNA expression after exposure to UCMS: LTE

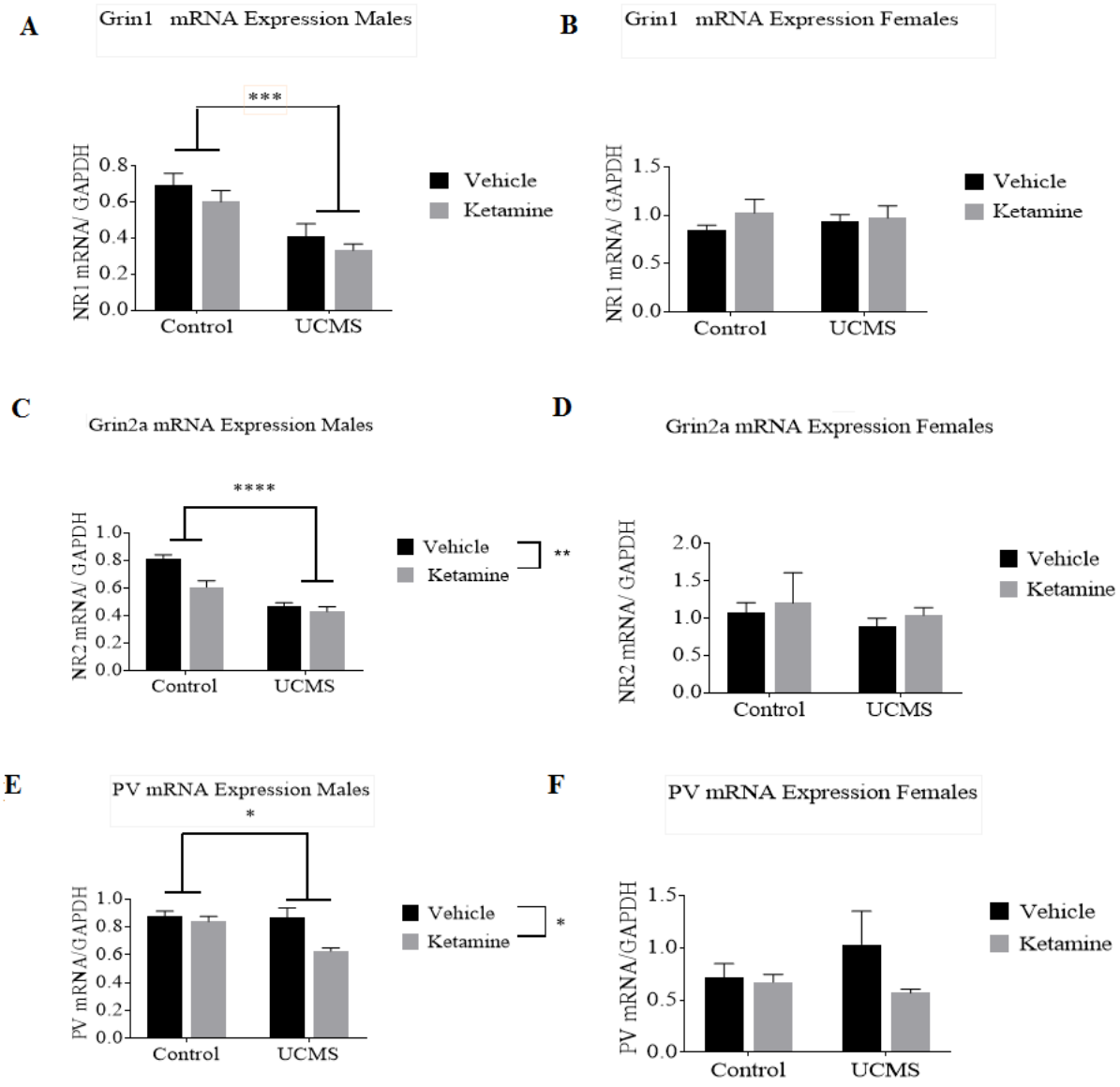

Figure 7: Grin 1(NR1) (p=0.0009- Fig 7A) expression in males following UCMS suggests stress induced general decrease expression of NMDA-r in male PFC. Analysis of Grin 2a (Fig 7C) reveal a similar decrease in NMDA-r expression in males (p<0.0001) which was strengthened by ketamine (p= 0.0031). PV was significantly decreased in UCMS males (p=0.0326- Fig 7E), an effect that was strengthened by ketamine in both experimental groups (p=0.00114). No significant effects of experimental groups (control, UCMS), or treatment (ketamine, vehicle) were observed in females.

Effects of ketamine on mRNA expression in STE subjects:

RT- qPCR examination of mRNA expression of Grin 1 reveals a decrease in Grin 1 expression in males who received the ketamine treatment (Fig. 8A; $t=2.355$, $df=9$, $p=0.043$). In females, however, no significant decreases or differences in mRNA expression were observed between the control and treatment groups. Analysis of Grin 2a in STE group reveals no difference in mRNA expression between control and ketamine treated males. Similarly, analysis of the same marker in females reveals no difference in mRNA expression between control and ketamine treated females. In examining mRNA expression of PV, no significant differences were observed in mRNA expression between treatments for both males and females. However, a trending reduction of PV expression was observed following ketamine treatment in males (Fig. 8K: $p=0.0600$).

Effects of ketamine on mRNA expression without exposure to UCMS: STE

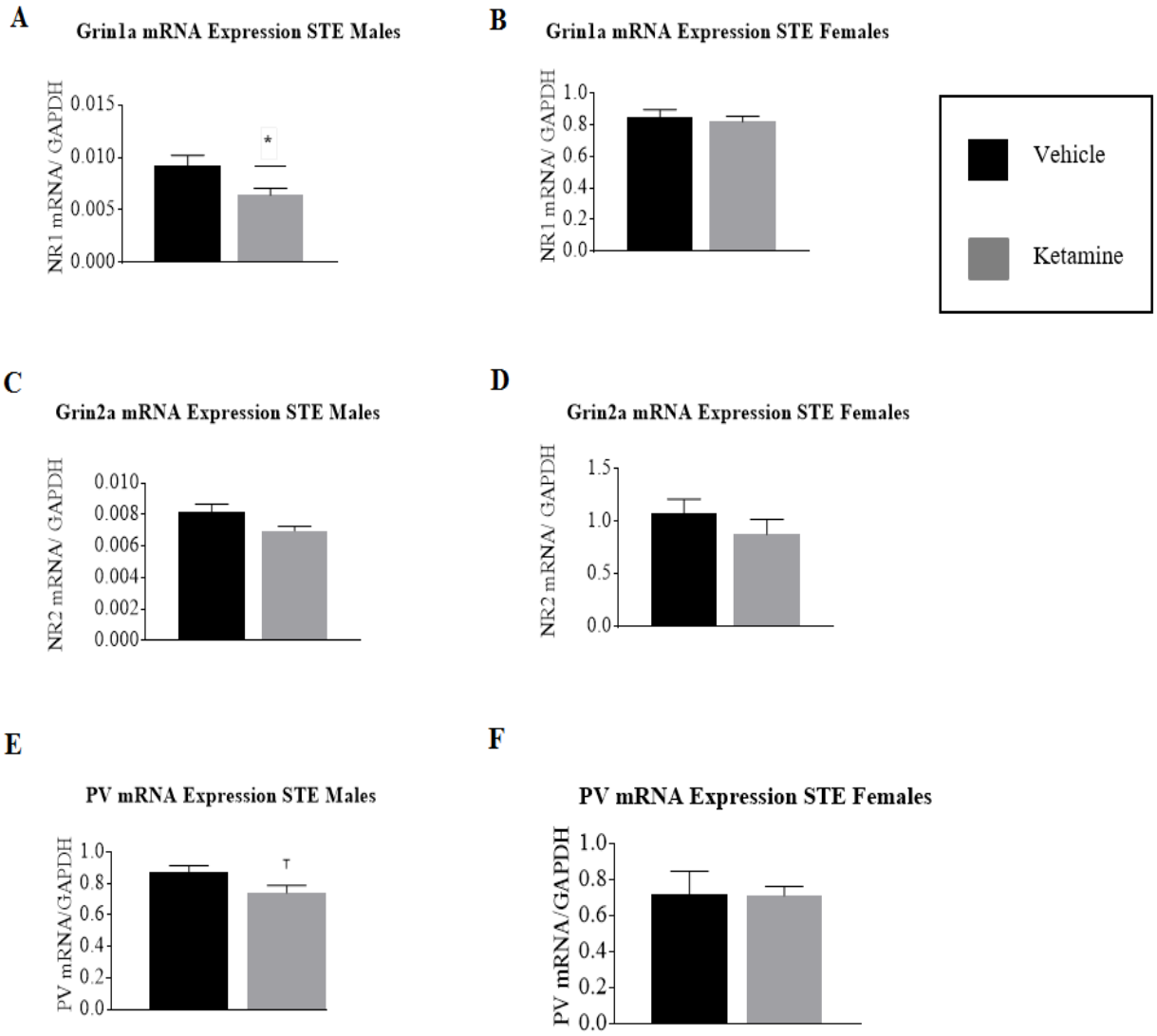

Figure 8: Analysis of Grin 1, Grin 2a and PV markers in the STE group reveal a decrease of NR1 in ketamine treated males (p=0.0430- Fig. 8G). No significant differences were observed in Grin 2a mRNA expression in males. No significant differences were observed in mRNA expression of PV between treatments though a trending reduction of PV expression was observed following ketamine treatment in males (p=0.0600- Fig. 8K). No significant effects of treatment (ketamine, vehicle) were observed in STE female mRNA expression of Grin 1, Grin 2a, and PV.

Discussion:

The results of this study confirm current Neuroscience knowledge regarding stress and its effects, and reveal novel information that further elucidates our understanding of the stress phenomena, and offers possible new directions and treatments for stress vulnerable populations. Neuroscience research supports that patients suffering from depression and/or anxiety typically display changes in their prefrontal cortex that are associated with some of the symptoms of these mood disorders (Drevets, 2000; Seminowicz et al.,2004; Treadway et al., 2015). This cooccurrence of prefrontal changes and anxiety-like and depressive-like behavior has also been observed in mice (Shepard et al. 2016). In our experiment, we also observed these phenomena.

Forced swim test data show UCMS induced the depressive-like behavior in both males and females. Previously conducted experiments have proven the validity of the FST in measuring depressive like behavior (Yankelevitch-Yahav et al., 2015) and have shown that exposure to 3-7 weeks of UCMS induces depressive like behavior in the forced swim tests, tail suspension test, and sucrose consumption test in male mice (Pothion et. al., 2004; Mineur et al., 2006; Farley et al., 2012) and more severe FST depressive like behavior in female mice (Shepard et. al, 2016). A paper published by Brachman et. al., (2016) recently revealed the potential of prophylactic ketamine to provide resiliency to depressive like behavior in male mice exposed to chronic social defeat. Our findings offer novel information as they, for the first time, reveal the ability of a 10mg/kg dose of ketamine administered prophylactically to rescue the depressive-like behavior in males, and not in females.

Secondly, our open field test data also confirmed current knowledge that females usually display more anxiety- like behavior following stress than males (Shepard et., 2016, Maeng et. al., 2010). We observed that though UCMS induced increased locomotion in both males and

females, females were found to display more significant anxiety like behavior. Importantly, we observed a trending effect of ketamine's ability to decrease hyperlocomotion in females. Conducting an experiment with an increased sample size will better clarify this trend and possibly reveal the ketamine's potential to decrease stress induced hyperlocomotion in female mice.

In our object recognition test, we observed a main effect of ketamine in decreasing male subjects' ability to discriminate between the familiar and unfamiliar object during testing day. Such results indicate ketamine induces impaired cognitive function in males. Experiments conducted in male rats have shown the administration of ketamine to lead to decreased habituation and inability to update spatial representations (Pitsikas & Boultadakis, 2009; Venâncio et. al., 2011). However, to our knowledge, we are the first to demonstrate that prophylactic ketamine administered at a dose of 10 mg/kg one week before a UCMS period induces cognitive deficits in male mice and not female mice.

Historically, research conducted examining the relationship between NMDA-receptors and ketamine has been focused on administration of ketamine after development of emotionality disorders. Findings of such research suggest that differential expression of NMDA-receptors in mood disorders are responsible for observed differential responses to ketamine (Dean B. et al., 2016). However, our data reveal that when administered prophylactically, ketamine alters the expression of NMDA-receptor proteins (Grin 1, Grin 2a), and alters the PV protein levels in male mice and not female mice.

In consideration of sex-dependent differences in vulnerability to stress induced emotionality disorders, examination of RT- qPCR data indicates molecular differences that might be responsible for these changes. These results indicate a more significant down regulation of

NMDA-receptors and PV cells in male subjects that could explain male resilience to stress induced depressive-like behavior. Since this decrease in NMDA-receptor and PV mRNA expression was not at all observed in females, it further supports that a lack of down regulation of NMDA-receptors in females, combined with a lack of down regulation of PV cells might explain the increased vulnerability to stress in females, that is not observed in males.

In the paper published by Coutellier Lab (Shepard et. al, 2016) female mice were found to have increased parvalbumin expression (PV) in the PFC following UCMS. This was associated with prefrontal hypoactivity and increased emotionality. Contrastingly, the male subjects displayed minor changes in emotionality following UCMS, with very little changes in their prefrontal PV expression. In this experiment, males were found to have significant decreased PV expression following UCMS. As such, this decrease in PV mRNA expression supports the lab's previously published findings regarding lowered levels of PV expression in males following UCMS. In analyzing the short-term effects of ketamine with our STE group, we see that even without exposure to stress, ketamine also significantly decreases Grin 1 mRNA expression. Additionally, a trending effect of its ability to reduce PV expression is observed before exposure to stress.

This novel information revealed by our experiment paves the way for more scientific research into the mechanism of stress. Our identification of a relation between prophylactic ketamine treatment, NMDA-r and PV expression and resilience to depressive-like behavior in mice advocates for the use of NMDA-r antagonists as a possible prophylactic treatment for male populations before exposure to stress. Of course, there exists many steps between basic science research and translating its findings to cater to the needs of human populations. However,

Neuroscience and Psychology scientists can begin exploring how this phenomenon can be translated to clinical research.

Though ketamine is an NMDA-receptor antagonist, it is known to have many other effects which are based on the dose at which it is administered (Idvall et al., 1979; Zarate et al. 2006; Murrough et al., 2013; Brachman et al., 2016). As such, this makes it a drug that can be easily abused if not properly regulated. Therefore, another development for future research stemming from our experiment is to explore other potential NMDA-receptor antagonists which might serve as better alternatives. This diversion will include investigation into the mechanism of NMDA-receptors; which might provide blue prints to produce synthetic, and healthier antagonists.

In conclusion, this experiment reveals novel information that elucidates our understanding of sex differences in resiliency to stress. Specifically, this experiment suggests stress induced depressive like behavior in males can be prevented via prophylactic administration of NMDA-receptor antagonists. As such, these results fail to reject the portion of our hypothesis that suggests a relationship between stress and NMDA-receptors. However, much remains to be discovered about the relationship between stress, NMDA-receptors, and PV cells. Although we did observe a decrease in PV cells in UCMS males which was strengthened by ketamine, administration of the NMDA-receptor antagonist did not rescue the stress induced phenotype in females. Exploring the specific interaction between NMDA-receptors, PV cells, and estrus cycles in females might further elucidate our findings. Estrogen receptors (estrogen receptor- beta) have been found to co-localize with PV cells in the cortex, amygdala, basal forebrain, and hippocampal formation of intact and ovariectomized adult rats (Blurton-Jones et al., 2002). Thus, further exploration of this colocalization specifically in the PFC of adult mice might explain our

observations. Our next step is to thus further understand the molecular mechanisms behind these phenomena via immunohistochemistry methods.

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